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14. ABSTRACT

We have developed ASK1 inhibitors for the reduction of ER stress and the treatment of ALS. These compounds were screened in cellular models of ALS and lead compounds were assessed in zebrafish models of ER stress. Two lead compounds were identified that are protective against ER stress-induced neuronal death. ALS mouse models were treated with compounds that modulate neuronal signaling and demonstrate favorable pharmacokinetics, however; no significant differences in age of symptom onset or survival were observed. Testing and development of these compounds is ongoing.

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DEVELOPING ER STRESS INHIBITORS FOR TREATING ALS

Introduction:

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder which specifically targets motor neurons in the spinal cord, brainstem, and cortex (*1*) resulting in muscle weakness, paralysis, and eventual death, typically within 2-5 years of disease onset (*2*). ALS affects 5 in 100,000 individuals with military veterans displaying an increased risk of acquiring this fatal illness (*3*). Treatment options for ALS are severely limited and result in minimal benefits with respect to morbidity and life span. While the exact cause of ALS is unknown, a prominent pathological feature of this disease is the upregulation of endoplasmic reticulum (ER) stress pathways in motor neurons of affected individuals. Apoptosis signal-regulating kinase 1 (ASK1) is a critical signaling molecule involved in the ER stress response and its activation is associated with motor neuron death in ALS models (*4*). As we have recently identified two classes of small molecule that inhibit the ASK1 pathway, the objective of this study is to design potent and selective inhibitors of ASK1 which are brain penetrant and display favorable pharmacokinetics. The scope of this study includes the evaluation of these newly developed inhibitors both *in vitro* and in cellular assays, as well as *in vivo* in animal models of ALS, with an ultimate goal of providing drug candidates for preclinical development.

Body:

The *Specific Aims* of this project are:

Aim 1: Design and synthesize small molecule modulators of ASK1 that are potent, selective, brain penetrant and systemically active *in vivo.*

Aim 2: Characterize small molecule modulators of ASK1 in assays measuring *in vitro* and cellular potency and selectivity.

Aim 3: Evaluate novel small molecule modulators of ASK1 using in vitro ADME/T and in vivo pharmacokinetic (PK) assays.

Aim 4: Evaluate novel small molecule modulators of ASK1 in a transgenic zebrafish model of ALS.

Results:

Specific Aim 1: Design and synthesize small molecule modulators of ASK1 that are potent, selective, brain penetrant and systemically active *in vivo*.

Year 1:

- Investigated the structure-activity relationships (SAR) around the benzodiazepinone screening hits SBI-0047641 and MLS-0292126
- Developed a general route for the synthesis of benzodiazepinone analogues that is efficient and that allows for introduction of diverse substituents into the scaffold
- Generated lead structures of additional scaffolds to complement our work in the benzodiazepinone series of molecules
- Synthesized three different scaffolds, triazoles (SBI-0405958), benzothiazoles (SBI-0629929), and imidazopyridines (SBI-0630156), and determined the representative activity for each series in our *in vitro* kinase assay

Year 2:

- Continued synthesis of analogues to investigate the structure-activity relationships (SAR) around the benzodiazepinone series of ER stress inhibitors
- Performed structural optimization on compounds active in ASK1 in vitro kinase assays
- Produced lead structures of additional scaffolds to complement our work in the benzodiazepinone series of molecules with a focus on pyrimidine-derived scaffolds

Year 3:

In the third year of the project we continued to investigate the structure-activity relationships (SAR) around the benzodiazepinone series of ER stress inhibitors. As noted in the previous progress reports, we identified the efficacious benzodiazepinone derivative MLS-0315763 following SAR studies around the original screening hits SBI-0047641 and MLS-0292126. Further SAR studies resulted in the identification of the highly efficacious benzodiazepinone derivative SBI-0636399 (Fig 1.1). In year 3 the SAR was expanded to explore additional structures in the series and the new analogues were tested for their ability to inhibit thapsigargin induced ER stress. A summary of the results are tabulated in Figure 1.2 below. As shown in Figure 1.2, several analogues (SBI-0636464, SBI-0636470, SBI-0636406, SBI-0636549) with promising neuroprotective activity were identified and characterized in the cellular assay.

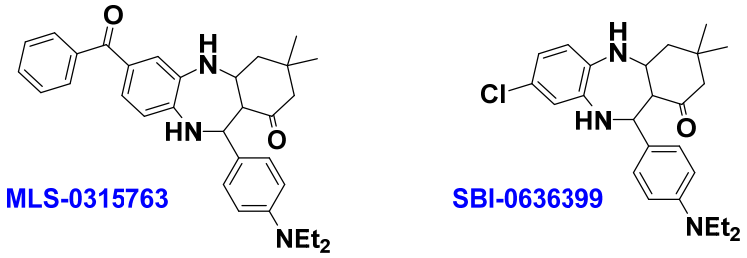
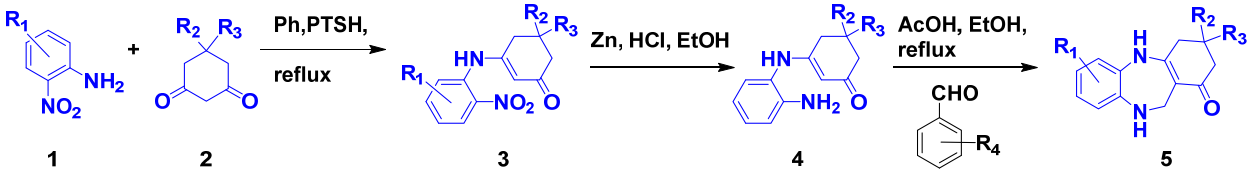


Figure 1.1. Structures of benzodiazepinone lead compounds MLS-0315763 and SBI-0636399.

The synthesis of analogues was accomplished using the established general route for the synthesis of benzodiazepinones that is efficient and allows for the introduction of diverse substituents into the scaffold (**Scheme 1**). Treatment of amino nitrobenzene **1** with 1,3-dione derivative **2** in benzene at reflux containing a catalytic amount of *p*-toluenesulphonic acid produces the nitroketone **3**. Reduction of the nitro group in **3** with zinc and ammonium chloride or hydrochloric acid produces the aminoketone **4**. Treatment of the amino ketone **4** with an aldehyde **5** in a solution of acetic acid and ethanol at reflux produces the desired benzodiazepinone analogues **5** in good overall yield (35 – 55 % for the entire sequence).



Scheme 1. Synthesis of benzodiazepinone derivatives.

Benzodiazepinone analogues synthesized as described previously were tested for their protective activity in cellular assays. To determine if these compounds protected against cell stress

in neuronal cells, we analyzed the ability of the benzodiazepinone derivatives to protect SH-SY5Y neuroblastoma cells from thapsigargin (TG) induced cell death (Fig 1.2).

Compound	EC_{50} (μM)	Compound	EC_{50} (μM)
SBI-0636463	17.71	SBI-0635694	34.58
SBI-0636464	8.61	SBI-0635703	20.81
SBI-0636465	>66	SBI-0635704	>66
SBI-0636466	10.23	SBI-0635705	>66
SBI-0636467	11.89	SBI-0635706	25.60
SBI-0636469	11.10	SBI-0636345	18.68
SBI-0636470	9.81	SBI-0636346	>66
SBI-0636471	>66	SBI-0636348	>66
SBI-0636472	12.01	SBI-0636351	32.99
SBI-0636480	22.78	SBI-0636352	>66
SBI-0636482	18.13	SBI-0636353	25.68
SBI-0636483	63.15	SBI-0636355	15.11
SBI-0636394	>66	SBI-0636360	57.42
SBI-0636396	>66	SBI-0636361	>66
SBI-0636397	13.63	SBI-0636362	>66
SBI-0636416	>66	SBI-0636390	14.42
SBI-0635631	40.30	SBI-0636395	17.44
SBI-0636392	23.85	SBI-0636401	14.00
SBI-0635625	11.44	SBI-0636406	7.87
SBI-0635626	23.96	SBI-0636408	10.41
SBI-0635628	14.10	SBI-0636410	12.30
SBI-0635629	13.52	SBI-0636413	11.22
SBI-0635630	>66	SBI-0636415	>66
SBI-0635681	11.18	SBI-0636546	9.90
SBI-0635683	15.57	SBI-0636547	10.41
SBI-0635684	>66	SBI-0636548	>66
SBI-0635685	>66	SBI-0636549	7.61
SBI-0635686	>66	SBI-0636550	>66
SBI-0635688	62.86	SBI-0636552	19.18
SBI-0638409	14.96	SBI-0636553	36.15
SBI-0638478	10.29	SBI-0636554	55.79
SBI-0638479	18.93	SBI-0636555	10.02
SBI-0638485	21.03	SBI-0636556	21.34
SBI-0638486	10.36	SBI-0637121	>66

Figure 1.2: **Benzodiazepinones protect SH-SY5Y cells from thapsigargin induced cell death.** EC_{50} values for benzodiazepinones active in single-dose experiments were determined by treating SH-SY5Y cells with 8 μM thapsigargin and various concentrations of the compounds. Cell viability was assessed following 4.5 h of treatment using ATPlite ^{TM} reagent (Perkin Elmer). Samples were read on a Perkin Elmer Viewlux plate reader in luminescence mode.

Specific Aim 2: Characterize small molecule modulators of ASK1 in assays measuring *in vitro* and cellular potency and selectivity

Year 1:

- Evaluated the potency of novel ASK1 modulators, generated in Aim 1, for the inhibition of ASK1 activity through the use of *in vitro* kinase assays

- Developed a cellular model of ER stress to determine the potency of novel compounds in human neuronal cell lines
- Identified the lead compound MLS-0315763, which displayed the highest potency for protection against thapsigargin induced ER stress in SH-SY5Y cells
- Developed assays using a motor neuron cell line expressing an ALS specific mutation to better recapitulate cellular conditions in ALS and determined that these cells are more susceptible to thapsigargin induced cytotoxicity
- Utilized this cellular model of ALS for further validation of lead compounds
- Assessed the ability of ASK1 modulators to influence the activity of cellular stress pathways downstream of ASK1 including c-Jun N-terminal kinase (JNK), p38MAPK, and C/EBP homologous protein (CHOP)
- Determined that the lead compound MLS-0315763 inhibits cellular stress affected by disruption of calcium homeostasis

Year 2:

- Analyzed the ability of the newly synthesized benzodiazepinone derivatives to protect SH-SY5Y neuroblastoma cells from thapsigargin (TG) induced cell death
- Discovered that one compound, SBI-0636399, demonstrated increased potency over our previously reported lead compound, MLS-0315763
- Determined that SBI-0636399, like MLS-0315763, protects NSC-34 cells expressing SOD1 proteins from thapsigargin induced cell death, inhibits calcium regulator-induced p38 MAPK activation, and prevents thapsigargin-induced p38 MAPK and JNK activation in H4 cells
- Ascertained that SBI-0636399 does not inhibit JNK activity in NSC-34 cells and furthermore inhibition of JNK and p38 do not protect against thapsigargin induced cell death, thus questioning the involvement of stress kinase inhibition in benzodiazepinone-mediated cytoprotection
- Demonstrated that benzodiazepinones potentiate SOC-mediated calcium influx
- Identified several novel compounds, structurally distinct from the benzodiazepinone series that are active in preliminary assays

Year 3:

In this year we have focused our cellular assays on the determination of the mechanism of action (MOA) of the benzodiazepinone series of compounds. In year 1 and year 2, we reproducibly found these compounds to be cytoprotective in neuronal cell lines challenged with the ER stressor thapsigargin. In particular, we found that the compounds were effective at preventing cell death in motor neuron cell lines expressing SOD1 mutations. However, although these compounds were originally characterized as ASK1 inhibitors, further studies have determined that reduction in stress kinase activity is dispensable for cellular survival. We then determined that the benzodiazepinone series was effective at reducing ER stress-mediated cell death only in response to thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) channels which initiates ER stress by preventing calcium reuptake by the ER. Using calcium mobilization assays we determined that active benzodiazepinone derivatives potentiate store-operated calcium (Ca^{2+}) entry (SOC) mediated entry, facilitating calcium mobilization in the cell.

These data led us to investigate whether our lead compounds protect cells from TG induced cell death through enhancing Ca^{2+}mobilization. Using specific channel inhibitors to eliminate the effect of benzodiazepinones on Ca^{2+}entry, we examined cell death in response to the ER stress inducers, thapsigargin and dithiothreitol (DTT) (Fig 2.1 and Fig 2.2). Neither YM-58483, a selective inhibitor of Ca^{2+}release-activated Ca^{2+} (CRAC) channels or EVP4593, an inhibitor of store-operated calcium (Ca^{2+}) entry (SOC), reduced benzodiazepinone mediated survival in NSC-34 cells. These

data suggest that calcium entry facilitated by CRAC and SOC channels does not enhance cellular survival in our assay.

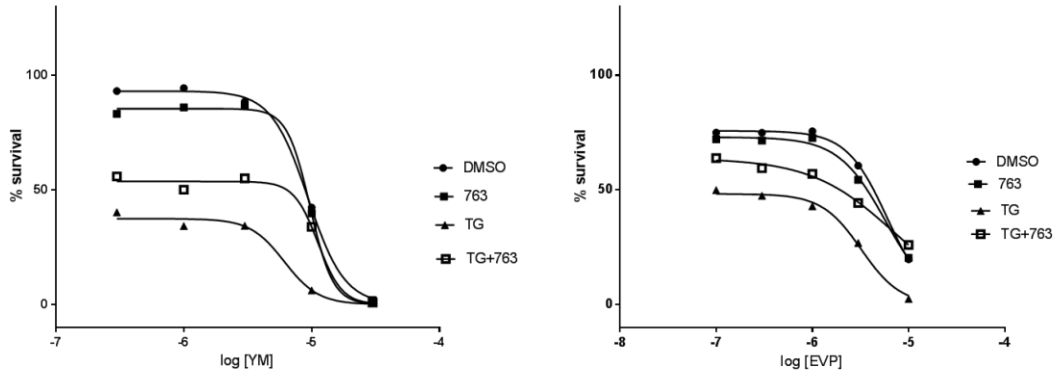


Figure 2.1: Dose response of YM-58483 or EVP4593 in NSC-34 cells treated with TG. NSC-34 cells were pretreated with DMSO or 25 µM of either MLS-0315763 or SBI-0636399 for 2 h prior to treatment with various concentrations of either YM-58483 (YM) or EVP4593 (EVP) in the presence or absence of 7.5 µM thapsigargin (TG) for 18 h. Cell viability was assessed using ATPlite™ (PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5.

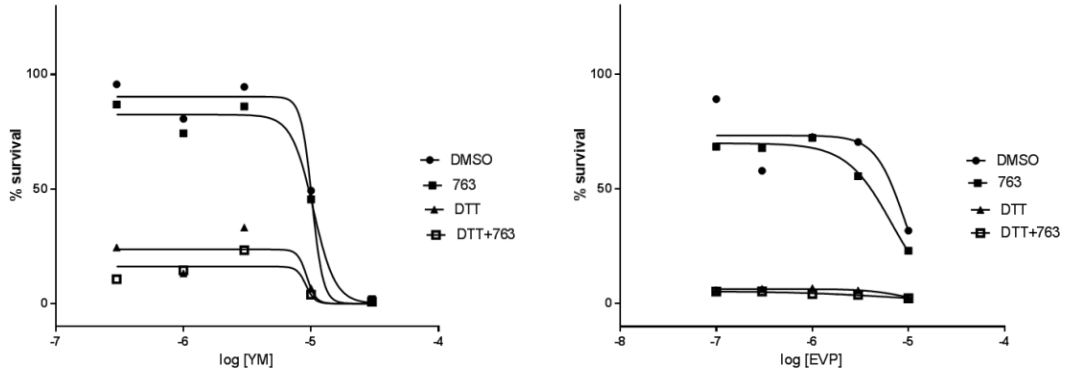


Figure 2.2: Dose response of YM-58483 or EVP4593 in NSC-34 cells treated with DTT. NSC-34 cells were pretreated with DMSO or 25 µM of either MLS-0315763 or SBI-0636399 for 2 h prior to treatment with various concentrations of either YM-58483 (YM) or EVP4593 (EVP) in the presence or absence of 3 mM dithiothreitol *(DTT)* for 18 h. Cell viability was assessed using ATPlite™ (PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5.

Next we sought to understand the influence of benzodiazepinones on alterations in calcium homeostasis, as disruptions in calcium regulation contribute to the cellular pathology of ALS (*5*). We used the calcium ionophore ionomycin and 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) (*6*) to alter cytoplasmic Ca^{2+} concentrations. We analysed whether our lead compounds MLS-0315763 and SBI-0636399 were able to protect against cellular death following exposure to these calcium dysregulating compounds. Benzodiazepinones were cytoprotective against insult with thapsigargin, but had no effect on cell death caused by either ionomycin or CDDO (Fig 2.3 and Fig 2.4).

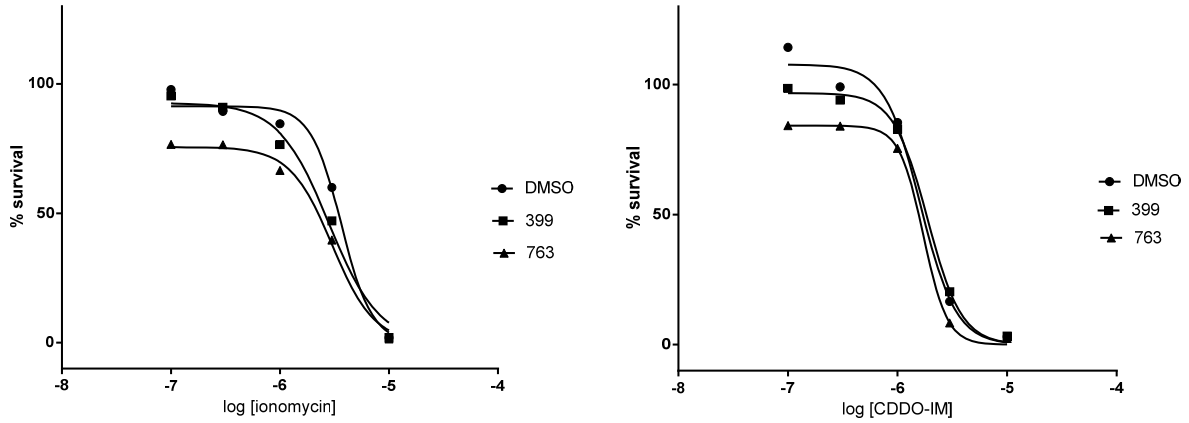


Figure 2.3: MLS-0315763 and SBI-0636399 do not protect against cell death mediated by ionomycin or CDDO. NSC-34 cells were pretreated with DMSO or 25 µM of either MLS-0315763 (763) or SBI-0636399 (399) for 2 h prior to treatment with various concentrations of either ionomycin or CDDO for 18 h. Cell viability was assessed using ATPlite ™(PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5.

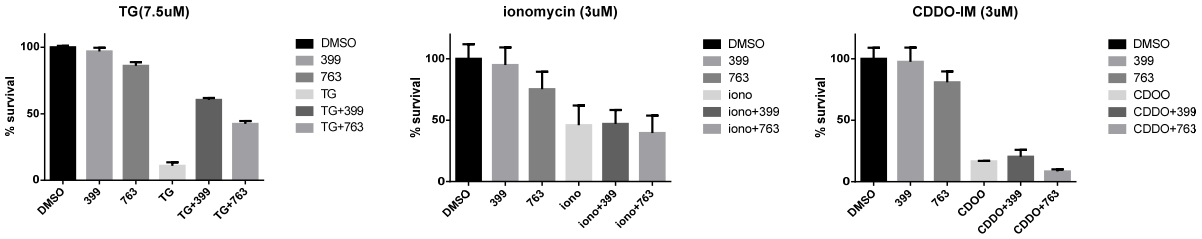


Figure 2.4: MLS-0315763 and SBI-0636399 protect against cell death mediated by thapsigargin, but not by ionomycin or CDDO. NSC-34 cells were pretreated with DMSO or 25 µM of either MLS-0315763 (763) or SBI-0636399 (399) for 2 h prior to treatment with either 7.5 µM thapsigargin (TG), 3 µM ionomycin, or 3 µM CDDO-IM for 18 h. Cell viability was assessed using ATPlite ™(PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5.

Finally, we analyzed spiperone, a psychotropic agent which has been demonstrated to increase intracellular calcium levels by facilitating calcium release from the ER (*7, 8*), similar to thapsigargin. We hypothesized that if the benzodiazepinones are cytoprotective due to their ability to restore calcium homeostasis following thapsigargin treatment then they would behave similarly to spiperone. First, we examined the movement of intracellular calcium in response to spiperone in the presence or absence of SBI-0636399 by performing calcium mobilization assays using a fluorescent dye to analyze intracellular calcium concentrations. Thus, NSC-34 cells were pretreated with SBI-0636399 and then stimulated with spiperone in calcium free media (Fig 2.5). Following spiperone stimulation (around 200 sec) we see an initial spike in intracellular calcium as ER calcium stores are released, then calcium is added to the extracellular media (around 800 sec) causing a second increase in intracellular Ca^{2+} as calcium enters the cell through store operated channels (SOC). However, unlike our previous studies with ligands that stimulate calcium movement from the ER, SBI-0636399 does not potentiate SOC mediated entry following stimulation with spiperone.

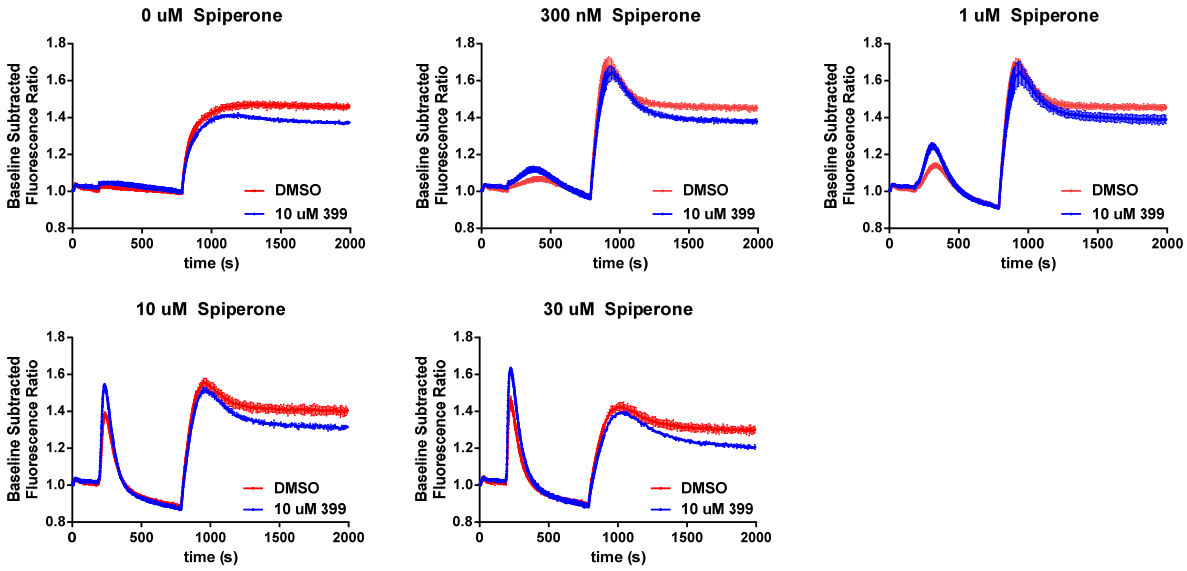


Figure 2.5: Benzodiazepinones do not potentiate SOC mediated Ca^{2+} entry in response to Spiperone mediated Ca^{2+} mobilization. NSC-34 cells were plated in 384 well black-walled clear bottomed poly-D-lysine coated plates (BD Biosciences) the day prior to assay. The following day, the medium was exchanged from the cells to calcium free assay buffer [calcium free Hanks' balanced salt solution (Invitrogen) containing 20 mM HEPES, pH 7.3] using an ELX-405 microplate washer (BioTek), leaving 20 µL/well, followed by the addition of 20 µL/well Fluo-4, AM (4.5 µM final concentration) indicator dye (Invitrogen; prepared as a stock in DMSO and mixed in a 1:1 ratio with Pluronic acid F-127) in assay buffer. Cells were incubated for 1 h at room temperature, and the dye exchanged to calcium free assay buffer using an ELX-405, leaving a concentration of 20 µL/well. SBI-0636399 *(399)* was prepared as a 2X stock in calcium free assay buffer (0.3% DMSO final concentration). Spiperone was prepared as a 5X stock in calcium free assay buffer. Calcium free assay buffer was supplemented with calcium at 10 times the final concentration to be assayed (1.8 mM final concentration). Cell plates and compound plates were loaded onto a kinetic imaging plate reader (FDSS 7000; Hamamatsu Corporation, Bridgewater, NJ). Appropriate baseline readings were taken (10 images at 1 Hz; excitation, 470 ± 20 nm; emission, 540 ± 30 nm) and test compounds were added in a 20 µL volume and incubated for approximately 2.5 min before the addition of 10 µL of spiperone to stimulate ER calcium release. After the addition of spiperone, data were collected for approximately an additional 10 min, followed by the addition of calcium containing HBSS as a calcium add-back to monitor SOC-mediated calcium flux.

Next we determined whether benzodiazepinones protect against spiperone mediated cell death similar to thapsigargin. However, benzodiazepinones were not only unable to rescue spiperone induced cell death, they enhanced spiperone mediated toxicity (Fig 2.6). Due to this finding we analyzed whether MLS-0315763 and SBI-0636399 exhibited cytoprotection in the presence of spiperone. As spiperone is a potent intracellular Ca^{2+} enhancer and releases Ca^{2+} from the ER through a PYK-Coupled PLC pathway (*7*), we also tested the cytoprotective properties of MLS-0315763 and SBI-0636399 in the presence of the nonselective PYK inhibitor, genistein. Neither treatment with spiperone or genistein blocked the ability of the benzodiazepinones to rescue thapsigargin-mediated cell death (Fig 2.7). Together these data provide strong evidence that the increase in SOC-mediated calcium entry in response to benzodiazepinone treatment does not contribute to cell survival.

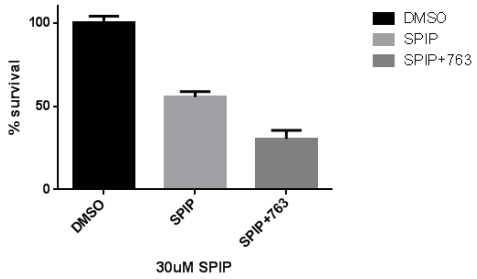
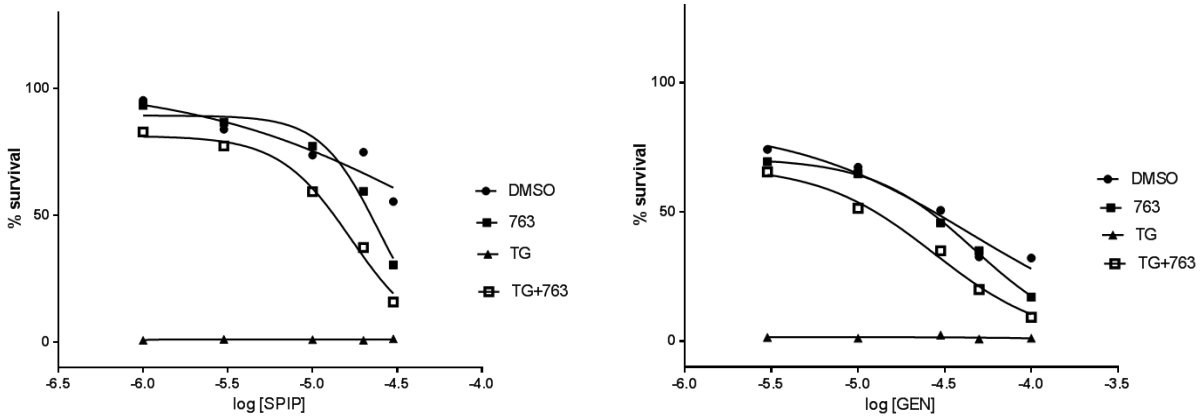


Figure 2.6: Benzodiazepinones are unable to rescue spiperone induced cell death. NSC-34 cells were pretreated with MLS-0315763 (763) or DMSO for 2 h prior to treatment with 30 µM spiperone (SPIP) for 18 h. Cell viability was assessed using ATPlite™ (PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5.

A



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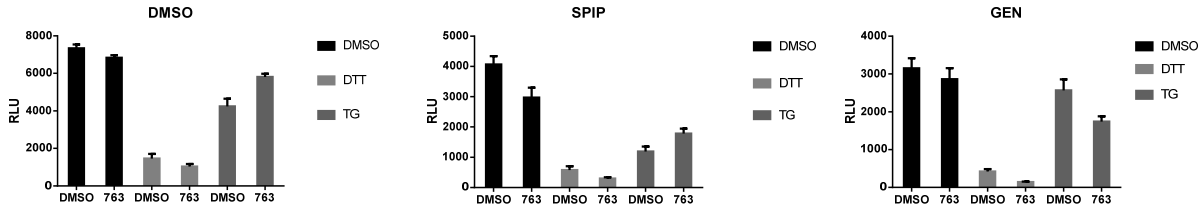

Figure 2.7: Neither spiperone nor genistein inhibit benzodiazepinone mediated survival in NSC-34 cells. A) NSC-34 cells were pretreated with DMSO or 25 µM MLS-0315763 (763) for 2 h prior to treatment with various concentrations of either spiperone (SPIP) or genistein (GEN) in combination with either DMSO or 7.5 µM thapsigargin (TG). Cells were incubated for 18 h. Cell viability was assessed using ATPlite™ (PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5. B) NSC-34 cells were pretreated with DMSO or 25 µM MLS-0315763 (763) for 2 h prior to treatment with either spiperone (SPIP) or genistein (GEN) in combination with either DMSO or 7.5 µM thapsigargin (TG). Cells were incubated for 18 h. Cell viability was assessed using ATPlite™ (PerkinElmer Life Sciences) and read on a BMG POLARstar Omega plate reader in luminescence mode. Data were analyzed using GraphPad Prism 5.

Thapsigargin has been identified as a potent activator of the signaling molecule, Ca^{2+}-independent phospholipase A2 (iPLA2) (*9-11*). PLA2s are esterases that hydrolyze phospholipids, resulting in the release of arachidonic acid and lysophospholipids and are implicated in inflammation and cellular injury (*12*). PLA2 upregulation has been demonstrated to be critical in triggering the inflammatory and neurodegenerative processes stimulated by deregulation of cyclin-dependent kinase 5 (Cdk5) which contributes to the pathology of many neurodegenerative disorders including AD and ALS (*13*). PLA2 isoforms have been demonstrated to upregulate lysophosphatidylcholine (LPC), a lipid mediator which initiates astrogliosis, neuroinflammation and subsequent neurodegeneration (*13*). Furthermore inhibitors of PLA2 enzymes are protective against ethanol-induced neurotoxicity (*14*). iPLA2 specifically has been implicated in promoting FasL (CD95L)-induced apoptosis (*15*) as well as activating the stress kinase p38 MAPK (*16*). Additionally iPLA2 can activate SOC channels and mediate store-operated Ca^{2+} entry (*9*).

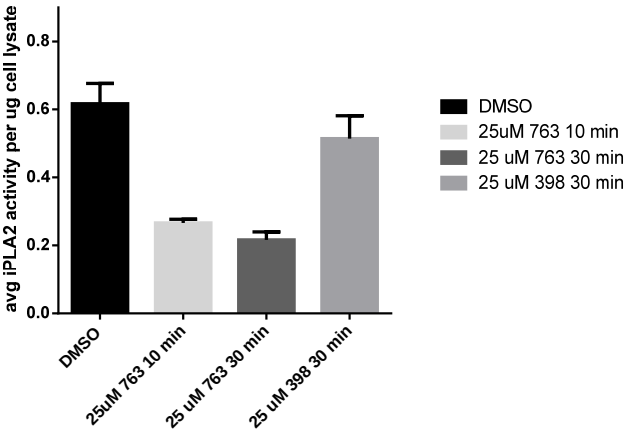

Figure 2.8: Benzodiazepinone treatment reduces iPLA2 activity. H4 cells were treated with DMSO, 25 µM MLS-0315763, or 25 µM SBI-0636398 (inactive control) for the indicated times. Cells were lysed in buffer containing 50 mM Hepes and 1 mM EDTA. iPLA2 activity was measured using the cPLA2 Assay Kit (Cayman Chemical) with modifications as described in Smani *et al*, 2003 (*9*). Lysates were incubated with the substrate, arachidonoyl thio-PC (1-hexadecyl-2-arachidonylthio-2-deoxy-*sn*-glycero-3-phosphocholine) for 1 h at 20 °C in a modified Ca^{2+}-free buffer. The reaction was stopped by the addition of 5,5--dithiobis(nitrobenzoic acid)for 5 min, and the absorbance was determined at 405 nm using a BMG POLARstar Omega plate reader. Protein concentrations of the lysates were analyzed using BCA reagent (Pierce).

Due to these data which suggest a role for upregulated PLA2 in cell death and degeneration, we hypothesized that benzodiazepinones may be protective against thapsigargin induced cellular death by influencing iPLA2 signaling. Therefore, we tested the activity of iPLA2 in cells treated with MLS-0315763 as compared to DMSO and an inactive compound (SBI-0636398). We observed a significant decrease in iPLA2 activity in benzodiazepinone treated cells (Fig 2.8). These data suggest that benzodiazepinones may protect against the thapsigargin induced death of neuronal cells through a reduction of iPLA2 activity. As PLA2 activity has been implicated in neurotoxicity, we plan to further investigate this potential role of the benzodiazepinone series in detail.

Specific Aim 3: Evaluate novel small molecule modulators of ASK1 using in vitro ADME/T and in vivo pharmacokinetic (PK) assays.

Year 1:

- Performed ADME/T assays on selected active compounds

Year 2:

- Performed ADME/T assays on additional active compounds

Year 3:

As we continue to test and develop the benzodiazepinone series as putative ALS treatments, we have also been pursuing new chemical entities for the treatment of neurodegenerative disorders. In order to test some of our novel compounds with potential for further development as therapeutics, we performed ADME/T assays as well as pharmacokinetic analysis in rats. We determined that two selected compounds, SBI-0646536 and SBI-0069359, had adequate plasma and microsomal stability as well as appreciable concentration in both the plasma and brain (Fig 3.1). These compounds were selected for treatment studies in SOD1 transgenic animals.

Compound ID	Plasma stability (1h)	Microsomal stability (1h)	Plasma * (μM)	Brain * (μM)	Brain:Plasma ratio
SBI-0646536	83%	58%	6.52 ± 0.54	0.22 ± 0.04	0.014
SBI-0069359	86%	45%	0.985 ± 0.168	4.818 ± 1.033	4.921

*performed at 1 h, 10mg/kg i.p.

Figure 3.1: ADME/T and PK analysis of novel compounds SBI-0646536 and SBI-0069359.

Specific Aim 4: Evaluate novel small molecule modulators of ASK1 in a transgenic zebrafish model of ALS.

Year 1:

- Established an *in vivo* zebrafish model of ER-stress by exposure of ER stress-inducing reagent Tunicamycin, which induces motor neuron degeneration in zebrafish, resulting in swimming aberrations and decreased survival.
- Developed an *in vitro* model of ER-stress to test Thapsigargin directly on motor neurons from a zebrafish line that expresses GFP specifically in all motor neurons.
- Determined that the lead compound, MLS-0315763 could alleviate Tunicamycin-caused swimming aberrations and prolong the survival of Tunicamycin-exposed zebrafish larvae.

Year 2:

- Determined that the lead compound SBI-0636399 alleviated tunicamycin-caused swimming aberrations in a dose-dependent manner and increased the survival rate of treated zebrafish, suggesting that SBI-0636399 is protective against neuronal damage and death in an *in vivo* model of ER stress.

- Examined a subset of compounds in primary neurons and ascertained that both MLS-0315763 and SBI-0636399 reversed TG mediated cell death in these cells.

Year 3:

As we further our understanding of the cytoprotective properties of the benzodiazepinones for the development of future analogues, we have initiated compound treatment of the SOD1 transgenic mouse line, Tg(SOD1G93A)1Gur/J with novel chemical entities from our lab that have demonstrated appreciable plasma and brain levels (Fig 3.1). Previous studies from our lab have

demonstrated that these compounds bind to receptors on neuronal surfaces and modulate neuronal signal transduction.

To test if these compounds have neuroprotective properties in ALS models, transgenic animals were purchased from Jackson Labs and bred, genotyped, and maintained according to all IACUC guidelines. Special considerations for ALS transgenic animals were followed as described in the Jackson Lab handbook "Working with ALS mice" *(17)*. Animals were given compound daily through intraperitoneal injection (i.p.) starting prior to symptom onset (around 8-9 weeks of age). Two readouts were measured, age of onset (as demonstrated by leg splay) and survival (animals were considered at end stage when they were unable to right themselves after 30 s). Treatment with either SBI-0646536 or SBI-0069359 did not have any appreciable effect on age of onset (Fig 4.1 and Fig 4.2). Following several weeks of treatment, daily compound injection with SBI-0069359 was observed to have secondary effects on the mice unrelated to ALS progression, therefore treatment with this compound was ceased.

Figure 4.1: Treatment with SBI-0069359 does not delay symptom onset in SOD1 transgenic animals. Animals were treated daily with 10 mg/kg SBI-0069359 i.p. or vehicle control. Symptom onset was identified by scoring leg splay and tremor. (n=10 control, n=10 SBI-0069359)

Treatment with SBI-0646536 continued until end stage, however, following analysis, this compound had little effect on survival (Fig 4.2). Despite the influence of these compounds on neuronal signaling, SBI-0646536 and SBI-0069359 did not delay symptom progression and SBI-0646536 did not enhance survival in ALS transgenic animals. Compound treatment and analysis of the SOD1 transgenic animals are ongoing.

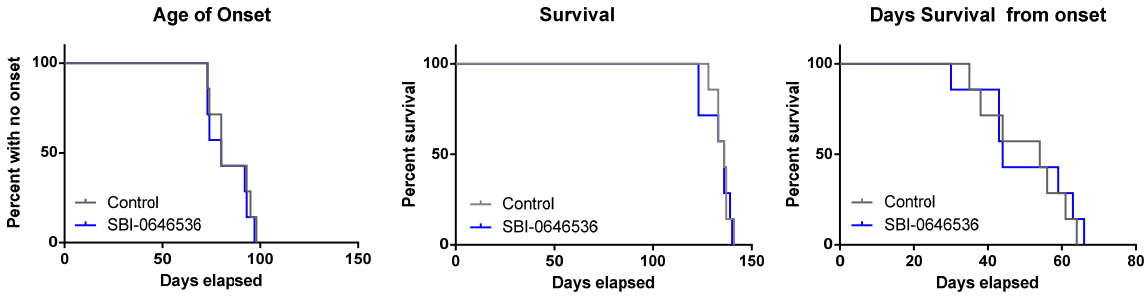

Figure 4.2: Treatment with SBI-0646536 does not delay symptom onset or prolong survival in SOD1 transgenic animals. Animals were treated daily with 10mg/kg SBI-0069359 i.p. or vehicle control. Symptom onset was identified by scoring leg splay and tremor. Animals were determined to be at end stage when they were unable to right themselves after 30 s. (n=7 control, n=7 SBI-0656536)

Key Research Accomplishments

Year 1:

- Established two chemical series that modulate ASK1 kinase
- Refined cellular assays for the analysis of ASK1 activity and stimulation of stress pathways
- Implemented a cellular model for ALS for use in compound screening assays
- Established a zebrafish model of ER stress that results in motor neuron degeneration
- Identified a lead compound with significant potency in multiple cellular assays, which protect motor neurons from damage and death in both cellular assays and zebrafish models of ER stress

Year 2:

- Identified the lead compound SBI-0636399 which has significant potency in numerous cellular assays and outperforms MLS-0315673 in some neuronal cell lines
- Established calcium mobilization studies to evaluate benzodiazepinone mediated protection against dysregulation in calcium homeostasis, a contributor to ALS pathology
- Identified SBI-0636399 as a potentiator of SOC mediated calcium influx which may function to alleviate calcium dysregulatory effects
- Determined that even though benzodiazepinones reduce stress kinase activity in specific cell lines, reduction in stress kinase activity alone has no effect on cellular survival
- Evaluated new compounds for ASK1 inhibition and identified several potential leads
- Used zebrafish model to test lead compounds in an animal model of ER stress

Year 3:

- Analyzed the effects of benzodiazepinone treatment on Ca2+ mobilization
- Determined that SOC-mediated capacitive calcium entry did not influence benzodiazepinone-mediated cytoprotection
- Established that Benzodiazepinone treatment reduces the activity of iPLA2, an enzyme that produces fatty acids and lysophospholipids that can increase inflammation and contribute to neurodegeneration
- Performed pharmacokinetic and ADME/T analysis on selected compounds and identified two compounds, SBI-0646536 and SBI-0069359, with adequate plasma concentrations and brain penetration for use in vivo studies
- Analyzed the effects of daily treatment with SBI-0646536 and SBI-0069359 on disease onset and survival in SOD1 transgenic mice

Additional Reportable Outcomes

Year 1:

- Published an invited review in the *Beilstein Journal of Organic Chemistry* entitled “Recent progress in the discovery of small molecules for the treatment of amyotrophic lateral sclerosis (ALS)”
- Established *in vivo* zebrafish model of ER-stress
- Created an H4 CHOP-luciferase reporter cell line

Year 2:

- Published a commentary in the Journal of Clinical Investigation (JCI) entitled, “Translational enhancers of EAAT2: therapeutic implications for neurodegenerative disease.” Limpert, A.S., Cosford, N. D. (2014) Translational enhancers of EAAT2: therapeutic implications for neurodegenerative disease, *J Clin Invest* 124(3), 964-7. PMID: 24569369.

Year 3:

- Published a manuscript in ACS Chemical Neuroscience, entitled, “Benzodiazepinone derivatives protect against endoplasmic reticulum stress-mediated cell death in human neuronal cell lines.” Zou H, Limpert AS, Zou J, Dembo A, Lee PS, Grant D, Ardecky R, Pinkerton AB, Magnuson GK, Goldman ME, Rong J, Teriete P, Sheffler DJ, Reed JC, Cosford ND (2015) Benzodiazepinone derivatives protect against endoplasmic reticulum stress-mediated cell death in human neuronal cell lines, *ACS Chem Neurosci* 6(3), 464-75. PMID:25544056

Conclusions:

In our goal to develop potent and selective inhibitors of ASK1 for use in the treatment of ALS, we have made substantial progress in the establishment and validation of two different chemical series, the establishment of a cell culture model for compound screening, and the development of a novel zebrafish model of ER stress for the testing of lead compounds. Furthermore, we have identified two compounds MLS-0315763 and SBI-0636399 that have been found to have significant potency in multiple cellular assays and protect against stress induced cell death in a motor neuron cell line expressing an ALS causing mutation. When tested in our zebrafish model, MLS-0315763 and SBI-0636399 alleviate swimming aberrations and prolong the survival of larvae. Importantly, MLS-0315763 and SBI-0636399 repeatedly protect motor neurons from stress induced cell damage and death. While stress kinase inhibition appears dispensable for MLS-0315763 and SBI-0636399-mediated cell survival, these compounds inhibit the activity of iPLA2, an enzyme implicated in inflammation and neuronal damage, indicating that further development of these compounds is warranted for the treatment of neurodegenerative disease.

Additionally, we have explored new chemical entities both for the inhibition of ASK1 and for the treatment of neurodegeneration. Of these compounds we selected two with appropriate pharmacokinetics for *in vivo* testing in SOD1 transgenic mice. Although neither SBI-0646536 nor SBI-0069359 appeared to be neuroprotective in these animals, our testing is ongoing.

Our work indicates that further development of ASK1 inhibitors is of significant value for the treatment of ALS. Future studies will be performed to create compounds with suitable properties for use in clinical trials.

The data that we have acquired over three years of investigation is an extremely valuable biomedical research product because it has enabled the refinement of compound development for the treatment of neurodegenerative disorders. Additionally, our newly synthesized compounds will provide potential therapeutics for ALS treatment, with the ultimate goal of developing a drug for use in human clinical trials.

Appendices:

None

Supporting Data:

None

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